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Glucose transport protein is structurally and immunologically related to band 3 and senescent cell antigen

(anti-idiotypic antibodies/two-dimensional peptide maps/immunoblots/cytochalasin B)

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ABSTRACT **Senescent cell antigen, a polypeptide that appears on the surface of senescent and damaged cells, has been shown to be derived from band 3. In the present study, the relationship between the as yet unidentified glucose transporter and senescent cell antigen is examined. Since cytochalasin B is a specific and potent competitive inhibitor of glucose transport in human erythrocytes, the glucose transport carrier was isolated by affinity chromatography on cytochalasin B-Sepharose 4B columns and eluted with D-glucose. This purification procedure is both a method of isolation and a functional assay for the glucose transporter. Purified glucose transporter gave a sharp, single band at M_r ≈60,000 when analyzed by NaDodSO$_4$/PAGE. Glucose transporter was identified in erythrocyte membranes by the immunoblotting technique, using both antibodies raised against purified glucose transporter and anti-idiotypic antibodies. Two-dimensional peptide mapping revealed substantial peptide homology between glucose transporter and senescent cell antigen. In addition, the glucose transporter shared peptide homology with band 3 and its defined proteolytic fragments located toward the carboxyl terminus of band 3. Peptide homology between glucose transporter and the M_r ≈41,000 cytoplasmic segment of band 3 that contains the amino terminus could not be demonstrated. Thus, glucose transporter appears to be part of or derived from band 3, and to share substantial peptide homology with senescent cell antigen.**

Senescent cell antigen is a glycosylated 4.5-region polypeptide that appears on the surface of senescent and damaged erythrocytes (1–14). [The "4.5 region" on NaDodSO$_4$/polyacrylamide gels contains polypeptides migrating between band 4.2 and band 5 (actin).] It is recognized by the antigen-binding (Fab) region (3, 4) of a specific IgG autoantibody in serum which attaches to it and initiates the removal of cells by macrophages (5). Although the senescent cell antigen was first demonstrated on the surface of senescent human erythrocytes (1, 2), it has since been demonstrated on the surface of lymphocytes, polymorphonuclear leukocytes, platelets, embryonic kidney cells, and adult liver cells (4). A molecule immunologically related to band 3, the molecule from which senescent cell antigen is derived, has been observed on all cells examined (15).

Senescent cell antigen has been "mapped" on the band 3 molecule by using topographically defined fragments of band 3 (16). Both binding of IgG eluted from senescent erythrocytes ("senescent cell IgG") to defined proteolytic fragments of band 3 in immunoblots and two-dimensional peptide mapping localized senescent cell antigen to a region on the extracellular (outer-surface) portion of band 3 that includes most of the ≈38-kDa carboxyl-terminal segment and about 30% of the ≈17-kDa anion-transport region.

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It was postulated that senescent cell antigen and glucose transporter were breakdown products of band 3 that were physiologically related (6). Both proteins migrate in the broad, uncharacterized 4.5 region of NaDodSO$_4$/polyacrylamide gels (4, 17). At least four polypeptides in this region stain with antibodies to band 3 (9, 10, 16). One of these polypeptides, the senescent cell antigen, appears to be derived from band 3 (6, 9, 10, 16). Band 3 is the major anion-transport polypeptide of the erythrocyte membrane, and, in addition, appears to be the binding site for the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (18), aldolase (19), and phosphofructokinase (20). In the present study, the relationship between the as yet unidentified glucose transporter that supplies energy to cells and the senescent cell antigen that signals the termination of a cell's life (1–14) is examined.

MATERIALS AND METHODS

Isolation of IgG from Senescent Erythrocytes and of Senescent-Cell Antigen. Senescent cell IgG was isolated as previously described (3, 4). Senescent cell antigen was isolated by affinity chromatography with senescent cell IgG conjugated to Sepharose 4B as described (4, 6).

Isolation of the Glucose Transporter. Since cytochalasin B is a specific and potent competitive inhibitor of D-glucose transport in human erythrocytes (21, 22), the glucose transport carrier was isolated by affinity chromatography on cytochalasin B-Sepharose 4B columns and eluted with D-glucose. This purification procedure is both a method of isolation and a functional assay for the glucose transporter (17, 23). Cytochalasin B (Calbiochem) was dissolved in dimethyl sulfoxide or absolute ethanol and conjugated to Sepharose 4B. Erythrocyte ghosts were prepared as described previously (10, 14, 15). NaOH-stripped membranes were solubilized with 0.1 M Tris/HCl, pH 8.0, containing 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diisopropyl fluorophosphate, and 0.1% NaDodSO$_4$ (10, 14, 15). After centrifugation, the supernatant was incubated overnight at 4°C with cytochalasin B-Sepharose. The Sepharose was removed by centrifugation and washed five times with 5 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, and 5 mM diisopropyl fluorophosphate. It was poured into a column and washed with 10 volumes of the same buffer in which the membranes were solubilized except that it contained 0.02% rather than 0.1% NaDodSO$_4$. After washing with 2 volumes of 1.0 M NaCl and 400 volumes of 0.15 M NaCl, bound material was eluted with 0.5 M D-glucose.

Preparation of Antibodies to the Glucose Transporter. Glucose transporter was isolated by affinity chromatography with cytochalasin B. While still bound to the column, the transporter was delipidated using water/ethanol/ether (1:1:4, vol/vol) and chloroform/methanol (2:1, vol/vol). The delipidated glucose transporter was specifically eluted from the affinity column with D-glucose and injected into rabbits,

using a biweekly injection schedule. Rabbits were bled 3 out of 4 weeks starting 1 week after the third injection. The IgG fraction was isolated by affinity chromatography on protein A-Sepharose.

Anti-idiotypic antibodies to the glucose transporter were prepared according to the method of Wassermann *et al.* (24). Briefly, antibodies were prepared against glucose by conjugating glucosamine to Sepharose 4B and injecting rabbits biweekly with the conjugate. Glucosamine rather than glucose was used because an amino group was necessary for coupling. After the third injection, glucosamine conjugated to bovine serum albumin as a carrier was injected on a biweekly schedule. The antisera were pooled, and antibodies to glucose were isolated by affinity chromatography on glucosamine-Sepharose 4B columns. Antibodies to glucose were eluted with glucose at pH 7.4. These antibodies then were injected into other rabbits to produce anti-idiotypic antibodies. The anti-idiotypic antibody rabbits were bled and the sera were adsorbed with bovine serum albumin and erythrocyte peripheral membrane proteins conjugated to Sepharose. The IgG fraction was isolated by affinity chromatography on protein A-Sepharose.

Antibodies to Band 3. Antibodies to purified band 3 were prepared in rabbits as described (9, 10, 14, 15).

Enzymatic Treatment of Erythrocytes. Fragments are referred to by the nomenclature of Steck *et al.* (25, 26). Washed erythrocytes were incubated overnight at 24°C in phosphate-buffered saline containing 1 mM ATP and α-chymotrypsin (200 μg/ml). α-Chymotrypsin specifically digests band 3, yielding fragments of M_r ≈55,000 and ≈38,000 (25, 26), designated CH-55 and CH-38, respectively (25). The CH-55 fragment appears to have a M_r closer to 60,000 in our experiments. Digestion was terminated by the addition of 5 mM diisopropyl fluorophosphate. Cells were washed four times with phosphate-buffered saline and processed in the same manner as described for intact erythrocytes.

The M_r ≈41,000 cytoplasmic segment (TR-41) of band 3 was produced by mild trypsin digestion of spectrin-depleted, NaOH-stripped inverted vesicles (27).

The M_r ≈19,000 intramembranous fragment (CH-TR-19), which includes CH-17, was produced by α-chymotrypsin treatment of intact red cells followed by treatment of spectrin-depleted inverted vesicles with trypsin (28). Proteolysis was terminated by the addition of 5 mM diisopropyl fluorophosphate. Membranes were washed twice with 5 mM sodium phosphate, pH 8.0/1 mM EDTA/1 mM EGTA/1 mM diisopropyl fluorophosphate.

NaDodSO$_4$/PAGE. Proteins were analyzed in three different polyacrylamide gel systems: 7% gels and 6–25% and 12–25% linear gradient gels. The discontinuous buffer system of Laemmli was used (29). M_rs given are approximate and can vary 5–10% depending on the PAGE system employed.

Immunostaining of Membrane Proteins. Immunoautoradiography was carried out either by the immunoblotting technique of Towbin *et al.* (30) with the modifications described previously (9, 10, 14–16) or by the gel-overlay method (10).

Two-Dimensional Peptide Mapping. Two-dimensional peptide maps were obtained by the method of Elder *et al.* (31), using the modifications described previously (16). Chromatograms were dried and exposed to Kodak X-Omat film in Dupont cassettes with Cronex Lightning Plus intensifying screens for 1–3 days at −80°C.

RESULTS

Identification of the Glucose Transporter by Using Antibodies Prepared Against It. Glucose transporter purified by affinity chromatography has a M_r ≈60,000 as determined by NaDodSO$_4$/PAGE (Fig. 1). Rabbit antibodies prepared against purified glucose transporter gave a very strong reaction with a polypeptide of M_r ≈78,000 (between bands 4.1 and 4.2) in NaDodSO$_4$/polyacrylamide gels of erythrocyte membranes. In addition, antibodies against purified glucose transporter reacted with band 3, minor bands and one major band in the 4.5 region, and polypeptides of M_r ≈40,000, 38,000, and 18,000 in immunoblots of erythrocyte membranes (Fig. 2, lane B). Antibodies to band 3 reacted with these same polypeptides, suggesting that they are immunologically related to band 3 (Fig. 2, lane A).

Identification of the Glucose Transporter Using Anti-Idiotypic Antibodies. According to the anti-idiotypic network theory of Jerne (32), injection of an antigen elicits antibodies to the antigen and, in addition, anti-idiotypic antibodies directed at the antigen-combining site (Fab hypervariable region) of the antigen-specific antibodies. It has been shown that production of antigen-specific antibodies against a ligand of a receptor results in anti-idiotypic antibodies that bind to the receptor itself (24, 33, 34). For example, immunization with insulin results in the production not only of antibodies to insulin (idiotypes) but also of antibodies that recognize the insulin receptor (anti-idiotypes) which are produced against the anti-insulin antibodies (35, 36). The anti-idiotypes sterically fit the combining sites of the idiotypes. Thus, the anti-idiotype appears to have a three-dimensional configuration similar to that of the ligand antigen (32, 35). Use of anti-idiotypes bypasses the requirement for a purified membrane molecule, since the initial antibody is made against the ligand and not the receptor (33).

Anti-idiotypic antibodies reacted with band 3, one polypeptide of apparent M_r ≈78,000, and one polypeptide (M_r ≈45,000) in the 4.5 region in NaDodSO$_4$/PAGE (Fig. 2, lane C). In addition, anti-idiotypic antibodies reacted with two faint bands in the 4.5 region (M_r ≈67,000 and 58,000). Antibodies to band 3 reacted with these same polypeptides (Fig.

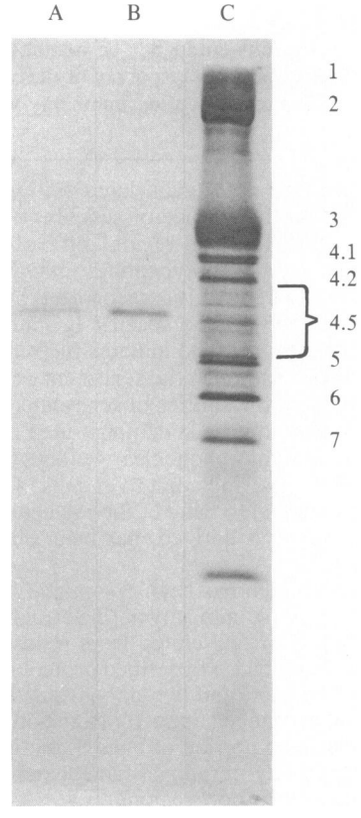

FIG. 1. NaDodSO$_4$/6–25% PAGE of affinity chromatography-purified glucose transporter (lane A) and senescent cell antigen (lane B) and of erythrocyte membrane proteins (lane C). Band designations are at right.

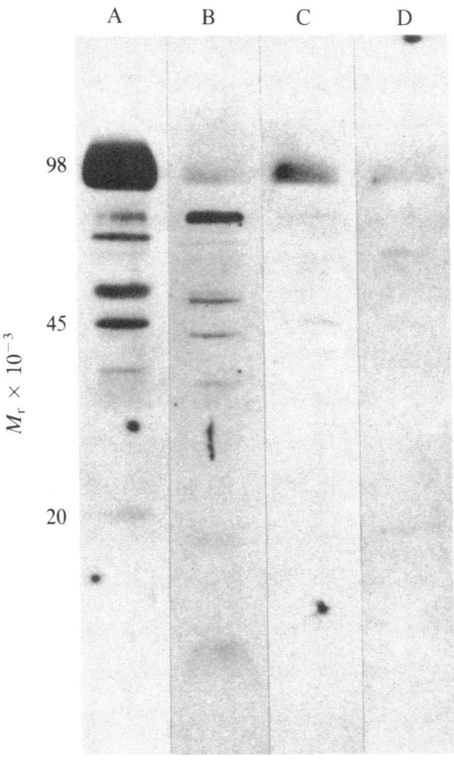

FIG. 2. Immunoblots of erythrocyte membrane proteins incubated with antibodies to band 3 (lane A), antibodies against purified glucose transporter (lane B), anti-idiotypic antibodies directed against the glucose receptor (lane C), and IgG eluted from senescent cells (lane D). Erythrocyte membrane proteins were separated on $NaDodSO_4$/12–25% polyacrylamide gels.

2, lane A). IgG eluted from senescent cells reacted with band 3, a 4.5 region polypeptide with a lower M_r than that of the glucose transporter (M_r ≈67,000), a polypeptide of M_r ≈45,000, and a polypeptide of M_r ≈18,000 (Fig. 2, lane D). Specific antibodies to band 3 also react with these polypeptides (Fig. 2, lane A).

Peptide Mapping Analysis of Band 3, Glucose Transporter, and Senescent Cell Antigen. Immunological data suggest that band 3 and glucose transporter are related. Therefore, comparative two-dimensional peptide mapping was performed to determine the extent of homology between the two polypeptides. Peptide homology between glucose transporter and senescent cell antigen was also evaluated because senescent cell antigen not only is derived from band 3 but also has been localized on the band 3 molecule. Comparative analysis of peptide maps (Fig. 3) revealed that glucose transporter (Fig. 3*B*) shares peptide homology with band 3 (Fig. 3*A*), the CH-38 carboxyl-terminal segment (Fig. 3*C*), and the CH-TR-19 anion-transport segment (Fig. 3*F*). The proportion of peptides in each map that appear in glucose transporter is as follows: band 3, 39–43% (27 peptides appear in glucose transporter/63–69 peptides in the map of band 3); CH-38, 63% (26/41); CH-TR-19, 67% (22/33); CH-55, 20% (10/50); TR-41, 3–5% (1 or possibly 2/40). The peptides present in the glucose transporter are present in the peptide map of band 3. Glucose transporter has 7–10 peptides that do not appear in CH-38 and 6 peptides that do not appear in CH-TR-19. However, all except 2 of the peptides in glucose transporter appear in the combined maps of CH-38 and CH-TR-19 as determined by superimposing the autoradiographs. These 2 glucose-transporter peptides that are not present in CH-38 combined with CH-TR-19 may be contributed by TR-41. Alternatively, they could result from technical factors discussed previously (16). For example, degradation of a polypeptide can produce an additional peptide at each end of a fragment where it was cleaved from band 3. Senescent cell antigen contains fewer peptides than glucose transporter, but all of the peptides present in the map of senescent cell antigen are present in the map of glucose transporter. Thus, glucose transporter (Fig. 4*A*) shares substantial peptide homology with senescent cell antigen (Fig. 4*B*), although the two maps are not identical.

DISCUSSION

Both the immunological and peptide mapping data indicate that glucose transporter is related to band 3. Glucose transporter appears to contain band 3 segments CH-38 and CH-17 and possibly several additional peptides. Thus, the calculated M_r of glucose transporter based on peptide mapping data is ≥55,000. Purified glucose transporter migrates at M_r ≈60,000 in PAGE, which is consistent with the peptide mapping data. However, both antibodies prepared against purified glucose transporter and anti-idiotypic antibodies to the glucose transporter react with a polypeptide of M_r ≈78,000 in erythrocyte membranes. The most probable explanation for the difference in apparent M_rs between purified glucose transporter (M_r ≈60,000) and the polypeptide labeled with antibodies in erythrocyte membranes is that degradation occurred during removal from membranes or during isolation procedures. It should be noted that both antibodies to glucose transporter and anti-idiotypic antibodies reacted with lower M_r polypeptides as well as the M_r ≈78,000 polypeptide in erythrocyte membranes. It is also possible that carbohydrates, glycolipids, or other membrane components could be associated with glucose transporter in membranes, thus increasing its apparent M_r, but that these associated components are removed or reduced during purification.

Glucose transporter is probably derived from or part of the band 3 molecule. However, mapping the peptides of glucose transporter to specific segments of band 3 is an approximation for reasons discussed elsewhere (16). Furthermore, if band 3 degradation occurs *in situ*, there is no reason to expect that *in situ* degradation would produce the same band 3 cleavage that we produce *in vitro* with enzymes.

Both glucose transporter and senescent cell antigen appear to be related to each other based on immunological and peptide mapping data. Both polypeptides appear to be located on segments toward the carboxyl rather than the amino terminus of band 3. Although senescent cell antigen appears to contain CH-38 and part of the CH-17 anion-transport segment of band 3 (16), glucose transporter appears to contain CH-38 and most of CH-17. Thus, glucose transporter appears to be larger than senescent cell antigen by peptide mapping analysis as well as antibody staining.

Neither purified senescent cell antigen nor glucose transporter appear to contain the cytoplasmic segment of band 3. It is possible that the cytoplasmic segment of band 3 (TR-41) behaves like a cytoskeletal protein with predominantly structural functions. It extends into the cytoplasm and appears to be the binding site for glyceraldehyde-3-phosphate dehydrogenase (37), aldolase (38), and band 2.1 (39). The cytoplasmic segment may also be more sensitive to proteolysis *in vivo* and/or *in vitro*. If this were the case, TR-41 could be degraded even though protease inhibitors are present throughout all procedures. Since TR-41 is not anchored in the membrane, it may be lost during the processing of membranes. The observation that breakdown products of band 3 increase with cellular aging suggests that degradation occurs *in vivo* (16).

The glucose transporter was isolated by affinity chromatography with cytochalasin B, a specific and potent competitive inhibitor of D-glucose transport (21, 22), and eluted with D-glucose. Thus, the method of isolation and purification—

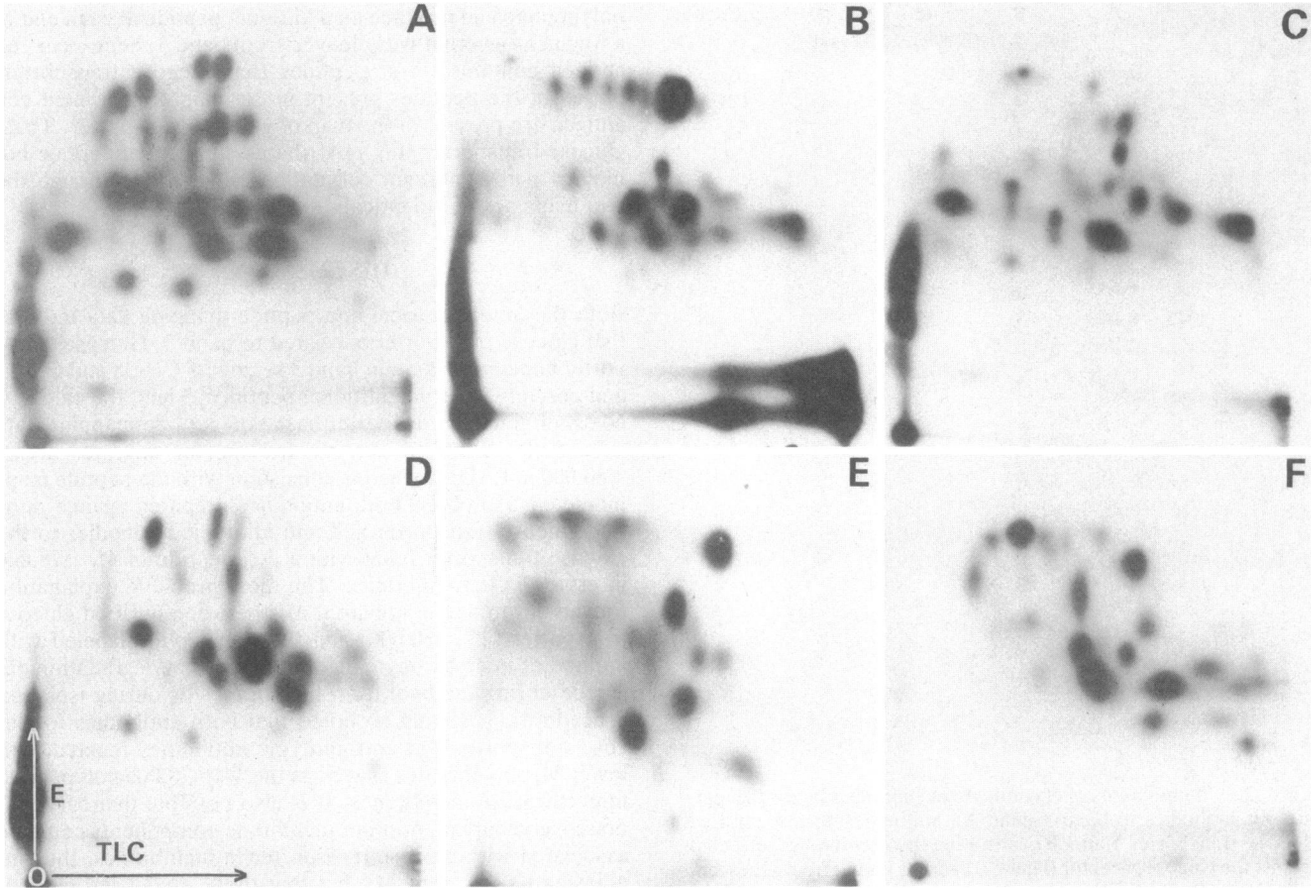

FIG. 3. Two-dimensional peptide maps of band 3, proteolytic products of band 3, and glucose transporter. (*A*) Band 3. (*B*) Glucose transporter. (*C*) CH-38. (*D*) CH-55. (*E*) TR-41. (*F*) CH-TR-19. This experiment was repeated 11 times on two different samples of glucose transporter and 4–5 different samples of the other polypeptides. Directions of electrophoresis (E) and TLC are shown, as is the origin (O) in *D*.

namely, cytochalasin B binding—is a functional assay that has been used to determine the presence of glucose transporter following other methods of isolation and/or enrichment (17, 23, 40). Antibodies prepared against the affinity-purified glucose transporter give a strong reaction with a M_r ≈78,000 polypeptide, a weaker reaction with band 3, and react with faint bands in the 4.5 region and polypeptides at M_r ≈38,000 and ≈18,000. In contrast, anti-idiotypic antibodies, which bypass the requirement for a purified receptor (33), bind to band 3 and its M_r 78,000 and 45,000 breakdown products. Anti-idiotypic antibody staining of band 3 is at least as strong as staining of the M_r 78,000 polypeptide. The differences in staining patterns between the antibodies to purified glucose transporter and the anti-idiotypic antibodies to glucose transporter are probably due to several factors. First, even though glucose transporter is treated to remove lipids, binding of associated lipid is reduced but not abolished by the procedures employed. Second, anti-idiotypic antibodies recognize the active site of the glucose transporter. Therefore, if the active site for glucose transport is a protein, then anti-idiotypes would "recognize" the protein and not associated carbohydrates or lipids. Polyclonal antibodies against

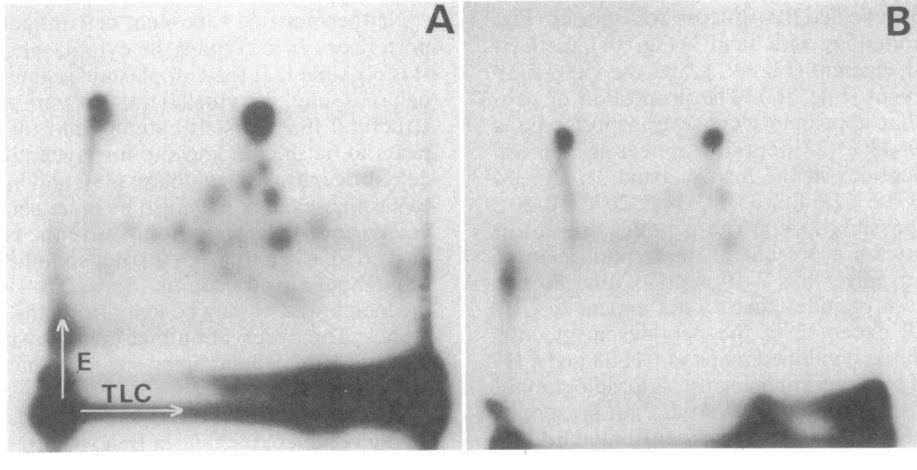

FIG. 4. Two-dimensional peptide maps of glucose transporter (*A*) and senescent cell antigen (*B*).

purified glucose transporter, on the other hand, would recognize any associated components and bind to them regardless of the molecule on which they appear. Since a number of glycoproteins may, for example, have similar sugar groups attached, the antibodies to purified glucose transporter may react with non-transport proteins on the basis of carbohydrate content rather than active-site configuration.

Early studies on the identification of the glucose transporter using affinity labeling or cytochalasin B binding suggested that glucose transport activity was attributable to band 3 (41, 42). This view was later supplanted by evidence indicating that an integral membrane glycoprotein of M_r 45,000–55,000 was probably the glucose transporter (17, 23). In the latter case, the glucose transporter was identified by [^{3}H]cytochalasin B binding to Triton X-100 extracts of erythrocyte proteins that were retarded on ion-exchange columns. Elution of the glucose transporter in these experiments did not require a pH or ion shift, and yielded a heterogeneous mixture which gave broad bands throughout the gels from the band 3 region to the tracking dye front (23). Antibodies prepared against glucose transporter obtained in this manner reacted with polypeptides of M_r 40,000–80,000 (23). A review of the PAGE data presented by Sogin and Hinkle (23) reveals that the trailing edge of antibody binding to glucose transporter, both in isolated preparations and in membranes, is in the band 3 ($M_r \approx 100,000$) region while the leading edge is in the band 6 region ($M_r \approx 35,000$). Zoccoli *et al.* (43) found that the M_r of the glucose transporter was 225,000 by using gel filtration and sucrose gradient centrifugation. They suggested that the solubilized cytochalasin B-binding complex contains one or two polypeptide chains (43), possibly dimers or aggregates of band 3.

The results presented here, which indicate that purified glucose transporter is structurally related to band 3, and that antibodies against affinity-purified glucose transporter react with band 3, a $M_r \approx 78,000$ polypeptide, faint bands in the 4.5 region, and lower M_r polypeptides, explain and reconcile the apparently disparate results obtained by other groups.

Yu and Steck (44) have suggested previously the possibility that band 3 itself is a set of closely related polypeptides that have several transport functions. The data presented here are consistent with this hypothesis and suggest that band 3 may be responsible for glucose as well as anion transport. In addition, the multifunctional band 3 molecule appears to be the origin for the senescent cell antigen (9–11, 16), a molecule whose appearance initiates a sequence of immunological events resulting in removal of "marked" cells (1–5).

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